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Preparation of Antigens and Immunoadsorbents Corresponding to the *Streptococcus* Group A Cell-wall Polysaccharide†

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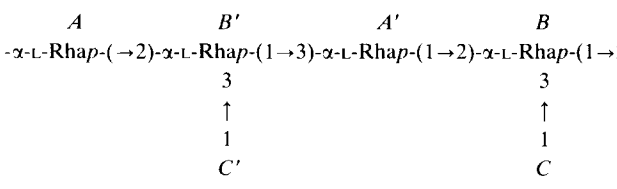
Abstract—The allyl glycosides of a tri-, penta- and hexasaccharide corresponding to the *Streptococcus* Group A cell-wall polysaccharide were coupled to solid or soluble supports to give immunoaffinity columns and neoglycoproteins, respectively. Cysteamine hydrochloride was added to the allyl glycosides and the resulting cysteamine adducts were used for subsequent coupling to linkers via the amine functionality. The tri- and penta- saccharide cysteamine adducts were coupled directly to the azalactone-derivatized 3M Emphase™ Biosupport Medium AB 1 to yield two affinity columns. The penta- and hexa- saccharides were coupled to bovine serum albumin or ovalbumin via the conjugate addition of the ε-amino groups of lysines on the proteins with the N-acryloylated sugars or the oligosaccharide-squarate adducts, derived in turn from the cysteamine adducts. The efficiency of the above methods is compared. Copyright © 1996 Published by Elsevier Science Ltd

Introduction

The β-hemolytic *Streptococcus* Group A is a common infective agent in humans, causing streptococcal pharyngitis (strep throat), some forms of pneumonia, and toxic shock-like syndrome.[1,2] When detected at an early stage, the infection may be readily treated with antibiotics, but when untreated or improperly treated it can act as a trigger of acute rheumatic fever, heart valve disease, or glomerulonephritis.[1,3] These complications are thought to result from an autoimmune reaction[4–8] in which antibodies directed against streptococcal antigens attack host tissues. In order to characterize these autoimmune responses, we have been pursuing a research program[9] to develop well defined immunodiagnostic reagents based on the *Streptococcus* Group A specific polysaccharide:[10,11]



Extensive syntheses[12–16] of oligosaccharides corresponding to the cell-wall polysaccharide as well as some of their corresponding soluble glycoconjugates[14] have been reported.

The methods to link oligosaccharides to proteins or to solid supports often follow similar strategies that have been previously reviewed.[17–20] A widely used coupling strategy is the reductive amination of reducing oligosaccharides[20,21] or aglycons containing aldehyde groups[22–25] with the free amino groups of a solid support or protein (ε-amino of lysines). Another popular method developed by Lemieux *et al.*[26] and modified by Pinto and Bundle[27] involves the preparation of 8-methoxycarbonyloctyl glycosides and their coupling to proteins via acyl azides. The latter method has the advantage of preserving the entire hapten and introducing a spacer-arm between the hapten and the protein thereby facilitating interactions between the sugar haptens and other macromolecules such as antibodies. Recently, this has also been achieved[28] via the coupling of a 5-aminopentenyl glycoside to an epoxy-modified polyacrylamide resin. Conjugate addition of the ε-amino groups of lysines to the double bonds of *N*-acryloylated oligosaccharides is also a widely used strategy to couple an oligosaccharide to a protein.[29] In this method, the amino group is introduced in the oligosaccharide either directly via the aglycon, e.g. by reduction of a *p*-nitrophenyl group, or via addition of cysteamine[30] to a deprotected allyl glycoside. More recently, a GDP-fucose analogue and a blood group A trisaccharide both containing a free amino group have been linked via a squarate group,[31] and the same strategy has been applied to the preparation of neoglycoproteins.[32]

The versatility of the allyl group as a temporary glycosidic protecting group which could be removed at the protected stage to allow further glycosylations has been extensively used in our laboratory for the convergent preparation of di- to hexasaccharides, as their propyl glycosides.[12–16,33] We have recently modified our synthetic strategy to allow the preparation of the corresponding deprotected allyl oligosaccharides.[33] We now report convenient procedures for the conjugation of these allyl oligosaccharides to both a solid support and soluble proteins for the preparation of affinity columns and soluble antigens, respectively.

†Dedicated with respect to Professor R.U. Lemieux.
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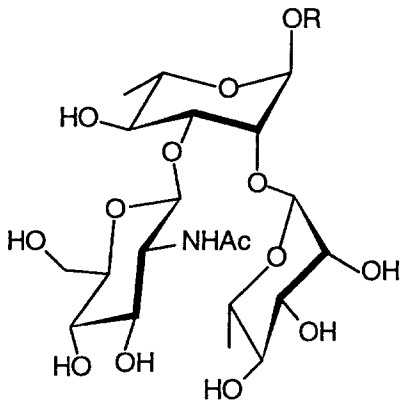
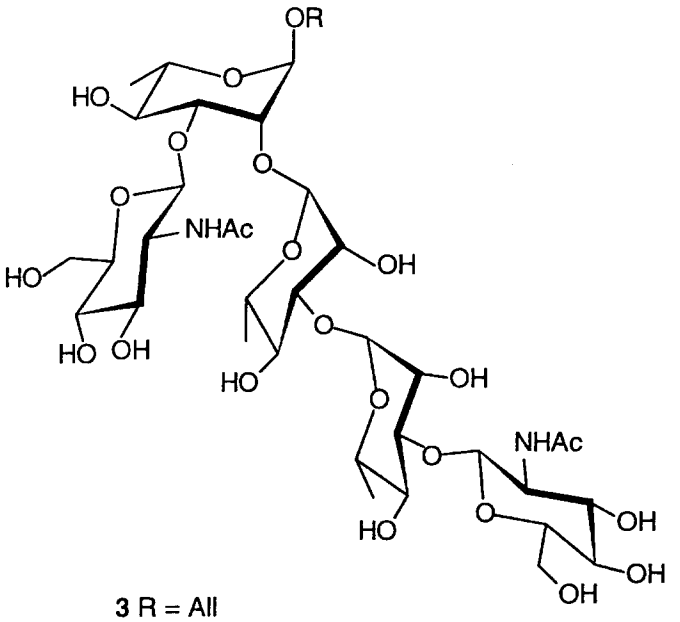


1 R = All

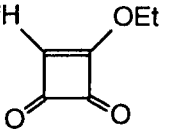
2 R = $(CH_2)_3S(CH_2)_2NH_2$

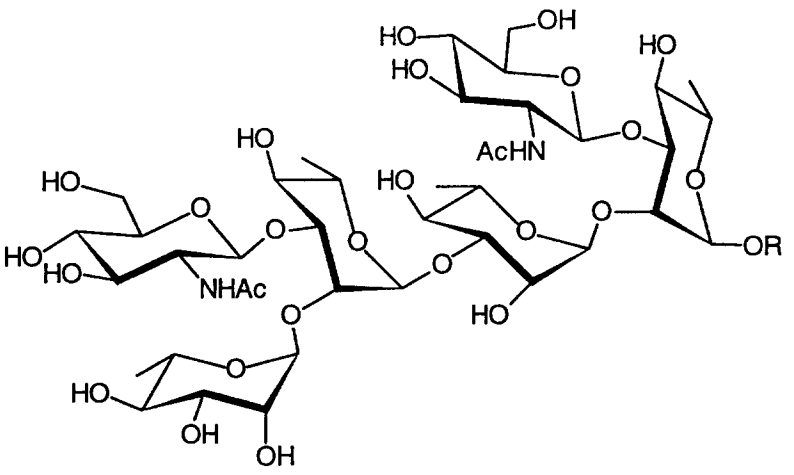


3 R = All

4 R = $(CH_2)_3S(CH_2)_2NH_2$

5 R = $(CH_2)_3S(CH_2)_2NHCOCHCH_2$

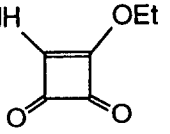
6 R = $(CH_2)_3S(CH_2)_2NH$ 



7 R = All

8 R = $(CH_2)_3S(CH_2)_2NH_2$

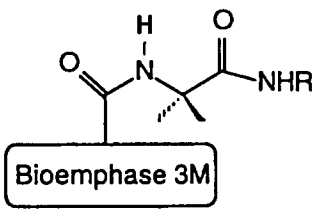
9 R = $(CH_2)_3S(CH_2)_2NHCOCHCH_2$

10 R = $(CH_2)_3S(CH_2)_2NH$ 

Results and Discussion

The convergent synthesis of the tri, penta- and hexasaccharides **1**, **3**, and **7** has been accomplished recently.[33] In order to introduce a spacer-arm between the oligosaccharide and the protein carrier or solid support, cysteamine hydrochloride was added to the allyl group of the oligosaccharides **1**, **3**, and **7**. The allyl glycosides were dissolved in a solution of cysteamine hydrochloride in a quartz tube and the mixtures were irradiated using a Hanovia UV-lamp. The adducts **2**, **4**, and **8** were purified from the excess cysteamine hydrochloride by gel permeation chromatography on a Biogel P2 column eluted with 0.25 M ammonium acetate and, after concentration, they were deionized on a column of Dowex (OH^-) ion exchange resin to give **2**, **4**, and **8** in 82–90% yield. The ^1H NMR spectral data (Table 1) indicated that the addition of cysteamine on the allyl group was quantitative under these reaction conditions.

The preparation of the immunoaffinity columns **11** and **12** was accomplished via the direct addition of the free amino group of the tri- or pentasaccharides **2** or **4** to the azalactone-bearing 3M Emphase™ Biosupport Medium AB 1 (UltraLink™ Immobilization Kit). The amount of non-incorporated sugar remaining in the solvent drained off the solid support was quantified by the method of Dubois *et al.*[34] and the amount of sugar incorporated on the solid support was then calculated by difference. Thus, the imunoaffinity columns **11** and **12** were shown to contain 22 μmol (≈2.6 μmol/ml of gel) of tri- or 16 μmol (≈1.8 μmol/ml of gel) of pentasaccharide per gram of gel, respectively. These results correspond to a 66 or 53% incorporation of the tri- and pentasaccharide, respectively.



11 R = trisaccharide

12 R = pentasaccharide

Coupling of the penta- and hexasaccharides to bovine serum albumin (BSA) or ovalbumin was first attempted via the conjugate addition[29] of the ε-amino groups of the lysines of the protein to the double bond of the *N*-acryloylated oligosaccharides **5** and **9**, derived from the cysteamine adducts **4** and **8**, respectively. In a preliminary test reaction, the *N*-acryloylation of the pentasaccharide **4** with an excess of acryloyl chloride in methanol containing triethylamine was followed by TLC. When no more starting material could be detected, the reaction mixture was concentrated and the crude product was de-ionized on a column of Dowex (OH^-). The ^1H-NMR (Table 1) spectrum of the crude compound showed that the *N*-acryloylated pentasaccharide **5** was pure. Subsequent *N*-acryloylations of the penta- or hexasaccharides **4** and **8** were conducted under the same conditions and the reactions were followed by TLC. When no more starting material was detected, the solvents were evaporated and the residues were freeze-dried from water to ensure complete removal of any residual methyl acrylate. The crude *N*-acryloylated penta- and hexasac-

13 BSA—[NH$(CH_2)_2$CONH$(CH_2)_2$S$(CH_2)_3$ -Pentasaccharide]$_{11}$

14 BSA—[NH$(CH_2)_2$CONH$(CH_2)_2$S$(CH_2)_3$ -Hexasaccharide]$_5$

15 Ovalbumin—[NH$(CH_2)_2$CONH$(CH_2)_2$S$(CH_2)_3$ -Pentasaccharide]$_1$

16 Ovalbumin—[NH$(CH_2)_2$CONH$(CH_2)_2$S$(CH_2)_3$—Hexasaccharide]$_7$

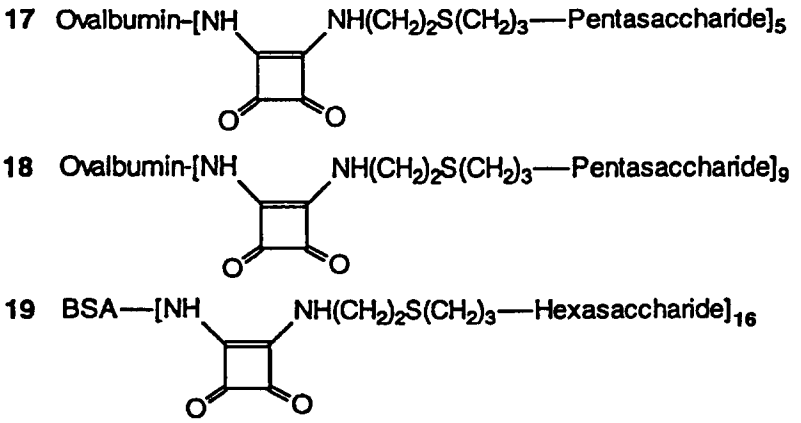


Table 1. ^{1}H NMR data[a] for compounds 2, 4, 5, and 8

Ring protons	2	4	5	8
1B	4.77	4.76	4.76	4.83
2B	4.12	4.13	4.13	4.14
3B	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.60
4B	3.61–3.44	3.60–3.36	3.60–3.34	3.60–3.34
5B	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.60
6B	1.24[b] (6.5)	1.24[b] (6.5)	1.24[b] (6.5)	1.27[b] (6.0)
1C	4.65 (8.5)	4.62[c] (8.5)	4.62[c] (8.5)	4.63[c] (8.5)
2C	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.63
3C	3.61–3.44	3.60	3.60	3.60
4C	3.44 ↑ multiplet ↓	↑ multiplet ↓	↑ multiplet ↓	↑ multiplet ↓
5C	3.35	3.36	3.34	3.34
6C	3.88	3.87	3.87	3.89
6′C	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.60
1A′	5.10	5.13	5.13	5.14 (1.5)
2A′	3.99 (3.0, 1.5)	4.03	4.03	4.03 (3.0, 1.5)
3A′	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.60
4A′	3.44–3.35	3.60–3.36	3.60–3.34	3.60–3.34
5A′	3.84–3.61	3.83–3.65	3.83–3.62	3.84–3.60
6A′	1.22[b] (6.5)	1.23[b] (6.0)	1.23[b] (6.0)	1.24[b] (6.0)
1B′		5.00 (1.0)	4.99	5.07 (1.0)
2B′		4.23 (3.0, 2.0)	4.23 (3.5, 1.5)	4.24
3B′		3.87	3.87	3.95 (9.5, 3.0)
4B′		3.60–3.36	3.60–3.34	3.60–3.34
5B′		3.83–3.65	3.83–3.62	3.84–3.60
6B′		1.25[b] (6.5)	1.25[b] (6.5)	1.25[b] (6.0)
1C′		4.66[c] (8.5)	4.66[c] (8.5)	4.69[c] (8.5)
2C′		3.83–3.65	3.83–3.62	3.84–3.60
3C′		3.60	3.60	3.60
4C′		↑ multiplet ↓	↑ multiplet ↓	↑ multiplet ↓
5C′		3.36	3.34	3.34
6C′		3.87	3.87	3.89
6′C′		3.83–3.65	3.83–3.62	3.84–3.60
1A				5.07 (1.0)
2A				4.00 (3.5, 1.5)
3A				3.84–3.60
4A				3.60–3.34
5A				3.84–3.60
6A				1.21[b] (6.5)

[a] The numbers in parentheses denote the coupling constants in Hz.
[b,c] Assignments may be interchanged.

charides **5** and **9** thus obtained were used directly for coupling with BSA or ovalbumin. The coupling reactions were accomplished in a 0.1 M carbonate buffer (pH 10) at 37 °C for 5–7 days to give the glycoconjugates **13-16** that were dialysed against water and lyophilized.

The sugar content of each of the glycoconjugates was assessed by the method of Dubois et al.[34] Similar measurements performed on BSA and ovalbumin showed that while BSA was, as expected, not glycosylated, the native oligosaccharide content of ovalbumin could not be neglected. The hapten contents of glycoconjugates **15** and **16** were corrected accordingly and the results for the glycoconjugates **13-16** are given in Table 2 (Entries 1–4). It is evident that the outcome of each reaction depends on both the nature of the oligosaccharide and the protein carrier. While 39% of the pentasaccharide **5** was incorporated on BSA, the same reaction on ovalbumin was unsuccessful (5% sugar incorporation). Opposite results were obtained with the hexasaccharide **9**, its incorporation on BSA being less (18%) than that on ovalbumin (37%). Interestingly, it was possible to recover the unreacted oligosaccharides **5** and **9** which were used in further coupling reactions.

In order to obtain better incorporation levels of the penta- and hexasaccharide on ovalbumin and BSA, respectively, we investigated an alternative conjugation method recently developed by Hällgren and Hindsgaul.[31] Reactions of the cysteamine adducts **4** and **8** with of 3,4-diethoxy-3-cyclobutene-1,2-dione (diethyl squarate) in methanol were thus investigated. TLC of the reaction mixtures showed the complete disappearance of the starting materials to give less polar, UV-absorbent and TLC-homogeneous compounds, assumed to be the oligosaccharide-squarate adducts **6** and **10**. The reaction mixtures were concentrated to dryness and the crude adducts **6** and **10** were coupled directly to ovalbumin and BSA, respectively. In a first attempt to prepare the ovalbumin–pentasaccharide glycoconjugate, the pentasaccharide **6** was left to react with the protein in 0.1 M carbonate buffer (pH 10) at 37°C for 7 days. Although better results were obtained than for the preparation of the glycoconjugate **15**, the level of incorporation of the pentasaccharide on ovalbumin was only 26% (Table 2, entry 5). The reaction was then attempted a second time with the same reagent and buffer concentrations but the mixture was left 7 days at room temperature. Under these conditions, the pentasaccharide **6** was incorporated with 45% yield on ovalbumin to give the glycoconjugate **18** containing nine hapten molecules per molecule of ovalbumin (Table 2, entry 6). The same reaction conditions were, therefore, used for the preparation of the BSA–hexasaccharide glycoconjugate **19**; the level of incorporation of the hexasaccharide was 59%, giving a glycoconjugate which contained 16 hapten molecules per BSA molecule.

In conclusion, allyl glycosides have been used to prepare two well defined immunoaffinity columns and a panel of glycoconjugates containing from 1 to 16 hapten molecules per protein carrier. The soluble glycoconjugates were prepared by coupling either the *N*-acryloylated or squarate adducts to BSA or ovalbumin. The latter method appears to be more

Table 2. Results of the coupling reactions of **5**, **6**, **9**, and **10** with BSA or ovalbumin

Entry	Activated sugar	Protein	T (°C)	Glycoconjugate	Sugar incorporation (%)	Hapten residues/ protein molecule
1	**5**	BSA	37	**13**	39	11
2	**5**	Ovalbumin	37	**15**	5	1
3	**9**	BSA	37	**14**	18	5
4	**9**	Ovalbumin	37	**16**	37	7
5	**6**	Ovalbumin	37	**17**	26	5
6	**6**	Ovalbumin	20	**18**	45	9
7	**10**	BSA	20	**19**	59	16

efficient in achieving a higher incorporation level of the oligosaccharide on the protein although more work is required to verify and quantify the various factors that control the efficiencies of the coupling reactions. In addition, the stability of the sugar–protein linkage via the squarate group remains to be investigated. The immunoaffinity columns will be used to purify monoclonal antibodies raised against the *Streptococcus* Group A specific cell-wall polysaccharide or to monitor autoimmune responses, and the soluble glycoconjugates will be used as immunizing antigens as well as screening agents for monoclonal antibodies in future studies.

Experimental

General methods

^{1}H NMR(400.13 MHz) and ^{13}C NMR(100.6 MHz) spectra were recorded on a Bruker AMX-400 NMR spectrometer for solutions in D_2O [internal standard: sodium trimethylsilyl-(2,2,3,3-tetradeutero)propionate].
Chemical shifts and coupling constants were obtained from a first-order analysis of one-dimensional spectra and assignments of proton and carbon resonances were based on COSY and ^{13}C–^{1}H heteronuclear correlated experiments. TLC was performed on precoated aluminum plates with Kieselgel silica gel 60 F_{254} (E. Merck) and detected with UV light and/or charred with a solution containing 1% $Ce(SO_4)_2$ and 1.5% molybdic acid in 10% aq H_2SO_4. Solvents were distilled and dried according to standard procedures,[35] and were evapd below 40 C under red. pres. Aminated oligosaccharides were purified by gel permeation chromatography on a Biogel P2 column (1.5 × 90 cm) eluted with a 0.25 M ammonium acetate aq soln. The 3M Emphase™ Biosupport Medium AB 1 (UltraLink™ Immobilization Kit) was purchased from Pierce, Inc. 3,4-Diethoxy-3-cyclobutene-1,2-dione (diethyl squarate) was purchased from Aldrich Chemical Co. A Hanovia UV lamp (200 W) was used for the irradiations. High resolution fast atom bombardment (FAB)-mass spectra were recorded in thioglycerol at 8 kV on a Kratos Concept H Magnetic Instruments mass spectrometer. The aminated oligosaccharides were characterized by FAB-mass spectra and high resolution NMR spectra. In a test reaction, the *N*-acryloylated pentasaccharide **5** was isolated and characterized by high resolution NMR spectroscopy; the compound decomposes on standing for prolonged periods. In subsequent preparations of **5** and the *N*-acryloylated hexasaccharide **9**, the compounds were not characterized but were used immediately in the coupling reactions. The oligosaccharide–ethyl squarate adducts were not isolated but were used directly in the coupling reactions to protein.

3-(2-Aminoethylthio)propyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (2). The allyl glycoside **1** (19.7 mg, 0.036 mmol) was dissolved in a soln of cysteamine hydrochloride in water (7.7 M, 0.2 ml) and the soln was irradiated for 1.2 h in quartz using a Hanovia UV-lamp. The mixture was dild with an ammonium acetate solution (0.25 M, 200 µl) and chromatographed twice by gel permeation chromatography on Biogel P2 to give the acetate salt of trisaccharide **2** that was contaminated by residual ammonium acetate. The crude trisaccharide [TLC, EtOAc:MeOH:H_2O, 6:3:1, R_f 0; *i*PrOH:NH_4OH(30% in water):H_2O, 7:2:1, R_f 0.3] was desalted and de-ionized on a Dowex (OH^-) column eluted with water. The cysteamine adduct **2** was obtained pure as an amorphous white powder upon freeze-drying (18.7 mg, 82%). NMR data for the ring protons are reported in Table 1. ^{1}H NMR (D_2O): δ 3.84–3.61 (m, 1H, OCH_2), 3.61–3.44 (m, 1H, OCH_2), 2.82 (m, 2H, SCH_2CH_2N), 2.62 (m, 4H, SCH_2CH_2N and $O(CH_2)_2CH_2S$), 1.99 (s, 3H, CH_3CON), 1.86 (m, 2H, $OCH_2CH_2CH_2S$). HRMS calcd for $C_{25}H_{46}N_2O_{14}S$ (M+H): 631.2748; found: 631.2734.

3-(2-Aminoethylthio)propyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (4). The allyl glycoside **3** (19.7 mg, 22 µmol) was dissolved in a soln of cysteamine-hydrochloride in water (7.7 M, 0.2 ml) and the soln was irradiated for 50 min in a quartz tube using a Hanovia UV-lamp. The pentasaccharide **4** [TLC, EtOAc:MeOH:H_2O, 6:3:1, R_f 0; *i*PrOH-NH_4OH(30% in water)-H_2O, 7:2:1, R_f 0.2] was purified on a Biogel P2 column and then desalted and de-ionized on a Dowex (OH^-) column eluted with water. The compound was obtained pure as an amorphous white powder upon freeze-drying (19.4 mg, 90%). NMR data for the ring protons are reported in

Table 1. ^{1}H NMR (D_2O): δ 3.83–3.65 (m, 1H, OCH_2), 3.60–3.36 (m, 1H, OCH_2), 2.85 (m, 2H, SCH_2CH_2N), 2.65 (m, 2H, SCH_2CH_2N), 2.62 (m, 2H, $O(CH_2)_2CH_2S$), 1.99 and 1.97 (2s, 2×3H, 2×CH_3CON), 1.86 (m, 2H, $OCH_2CH_2CH_2S$).HRMS calcd for $C_{39}H_{69}N_3O_{23}S$ (M+H): 980.4121; found: 980.4210.

3-(2-Acrylamidoethylthio)propyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (5). The pentasaccharide **4** (1.9 mg, 1.9 μmol) was dissolved in MeOH (0.5 ml), and NEt_3 (1.3 μl, 5 eq.) followed by acryloyl chloride (0.5 μl, 3 eq.) were added to the solution. The mixture was stirred for 15 min at room temperature. TLC using EtOAc:MeOH:H_2O, 6:3:1 as eluent showed that the reaction was not complete (R_f 0→0.4). More triethylamine (2×1.3 μl) and acryloyl chloride (2×0.5 μl) were added to the mixture and, after a few min at room temperature, TLC showed that the reaction was complete, giving a single UV-absorbent homogeneous spot (R_f 0.4). The solvents were evapd and the residue dissolved in 200 μl MeOH. The mixture was passed through a column (0.5 ml) of Dowex (OH^-) and eluted with MeOH. Fractions 1–7 (0.5 ml) were combined, concd, and the residue freeze-dried from water to give the pure pentasaccharide **5** (1.2 mg, 60%), as assessed by its ^{1}H NMR spectrum (Table 1).

Alternatively, the pentasaccharide (4.2 mg, 4.3 μmol) in MeOH (1 ml) was treated for 15 min with NEt_3 (2.5 μl, 4 eq.) and acryloyl chloride (1 μl, 3 eq.) at room temperature. TLC showed that the reaction was complete; solvents were evapd and the residue was freeze-dried to ensure complete removal of methyl acrylate. The crude acryloylated pentasaccharide was used directly for coupling with bovine serum albumin (BSA) or ovalbumin to give the glycoconjugates **13** and **15**, respectively.

NMR data for the ring protons are reported in Table 1. ^{1}H NMR (D_2O): δ 6.23 (dd, 1H, J=17.0 and 10.0 Hz, CH_2=), 6.14 (dd, 1H, J=17.0 and 1.5 Hz, CH_2=), 5.72 (dd, 1H, J=10.0 and 1.5 Hz, COCH=), 3.83–3.62 (m, 1H, OCH_2), 3.60–3.34 (m, 1H, OCH_2), 3.60–3.34 (m, 2H, SCH_2CH_2N), 2.70 (t, 2H, J=6.5 Hz, SCH_2CH_2N) 2.64 (m, 2H, $O(CH_2)_2CH_2S$), 1.98 and 1.97 (2s, 2×3H, 2×CH_3CON), 1.85 (m, 2H, $OCH_2CH_2CH_2S$).

3-(2-*N*-(3,4-dione-2-ethoxycyclobutene)aminoethylthio)-propyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (6). The pentasaccharide **4** (4.5mg, 4.6 μmol) was dissolved in freshly distilled MeOH (1 ml) and a soln (10 μl/ml, 68 μl, 1 eq.) of 3,4-diethoxy-3-cyclobutene-1,2-dione (diethyl squarate) in freshly distilled methanol was added. The reaction mixture was left standing at room temperature for 1 h, after which time TLC (EtOAc:MeOH:H_2O, 6:3:1) showed that the starting amine had been quantitatively converted to one homogeneous spot which was assumed to be the pentasaccharide **6** (R_f 0→0.35). The reaction mixture was concd to dryness and the pentasaccharide **6** was used without further purification in the coupling to ovalbumin to prepare the glycoconjugate **17**. A second aliquot of the pentasaccharide **4** (2.35 μmol) was treated with diethyl squarate under the same conditions described above and the product was used for the preparation of the glycoconjugate **18**.

3-(2-Aminoethylthio)propyl-3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (8). The 3-(2-aminoethylthio)propyl glycoside **8** was prepared from the allyl glycoside **7** (20.3 mg, 0.019 mmol) as described for the preparation of the pentasaccharide **4**. The hexasaccharide **8** [TLC, EtOAc:MeOH:H_2O, 6:3:1, R_f 0; *i*PrOH:NH_4OH(30% in water):H_2O, 7:2:1, R_f 0.2] was obtained pure as an amorphous white powder upon freeze-drying (17.9 mg, 82%). NMR data for the ring protons are reported in Table 1. ^{1}H NMR (D_2O): δ 3.84–3.60 (m, 1H, OCH_2), 3.60–3.34 (m, 1H, OCH_2), 2.86 (m, 2H, SCH_2CH_2N), 2.70–2.59 (m, 4H, SCH_2CH_2N and $O(CH_2)_2CH_2S$) 1.99 and 1.98 (2s, 2×3H, 2×CH_3CON), 1.87 (m, 2H, $OCH_2CH_2CH_2S$). HRMS calcd for $C_{45}H_{79}N_3O_{27}S$ (M+H): 1126.4670; found: 1126.4672.

3-(2-Acrylamidoethylthio)propyl 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (9). The hexasaccharide **8** (4.8 mg, 4.3 μmol) in MeOH (1ml) was treated for 30 min with NEt_3 (2.5 μl, 4.2 eq.) and acryloyl chloride (1 μl, 2.9 eq.) at room temperature. As TLC (EtOAc:MeOH:H_2O, 6:3:1) showed some remaining starting material (R_f 0→0.3), more NEt_3 (1.3 μl) and acryloyl chloride (0.5 μl) were added and the reaction mixture was left for an additional 0.5 h at room temperature. TLC showed that the reaction was complete; solvents were evapd and the residue freeze-dried to ensure complete removal of residual methyl acrylate. The crude acryloylated hexasaccharide **9** was used directly for coupling with BSA to give the glycoconjugate **14**. Acryloylation of another aliquot of hexasaccharide **8** (2.4 mg, 2.13 μmol) under the same conditions gave an additional sample of acryloylated hexasaccharide **9** that was used directly for coupling with ovalbumin to give the glycoconjugate **16**.

3-(2-*N*-(3,4-dione-2-ethoxycyclobutene)aminoethylthio)propyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(3-*O*-(3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-*O*-(α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (10). The hexasaccharide **8** (2.4 mg, 2.1 μmol) was treated with diethylsquarate as described for the preparation of the pentasaccharide **6**. After 3 h at

room temperature TLC (EtOAc:MeOH:H_2O, 6:3:1) showed that the starting amine had been quantitatively converted to one homogeneous spot which was assumed to be the hexasaccharide **10** (R_f 0→0.29). The reaction mixture was concentrated to dryness and the hexasaccharide **10** was used without further purification in the coupling to BSA to prepare the glycoconjugate **18**.

Preparation of the immunoaffinity columns 11 and 12

The trisaccharide **2** (1.6 mg, 2.5 μmol) and the pentasaccharide **4** (2.2 mg, 2.2 μmol) were each dissolved in a carbonate–citrate buffer (0.1 M $NaHCO_3$, 0.6 M sodium citrate, 700 μl, pH 9) and 3M Emphase™ Biosupport Medium AB 1 derivatized UltraLink (75 mg) was added to each soln. The slurries were shaken overnight at room temperature and transferred to 2 ml syringes equipped with teflon filters. The columns (≈650 μl) were washed with water (10 ml) and the first 5 ml of eluates were saved for quantitative analyses of the non-incorporated sugars. Quenching buffer (3M ethanolamine, pH 9) was added (2 ml per column) to the columns that were stoppered and shaken during 3–5 h at room temperature. The columns were drained and washed successively with water (10 column vols), 1 M NaCl (5 column vols) and a 80% aq. EtOH soln (10 column vols). The columns were stored at 4 °C in 80% aq. EtOH until use for immunoaffinity purifications. Quantitation of non-incorporated sugar was accomplished using the method of Dubois et al.[34] The level of sugar incorporation for each column was then assessed by difference. The immunoaffinity column **11** contained 22 μmol of trisaccharide/g of gel (≈2.6 μmol/ml of gel), corresponding to a 66% incorporation of the trisaccharide. The immunoaffinity column **12** contained 16 μmol of pentasaccharide/g of gel (≈1.8 μmol/ml of gel), corresponding to a 53% incorporation of the pentasaccharide.

Preparation of the BSA-glycoconjugates 13 and 14

BSA (10.2 mg in both cases) was dissolved in a 0.1 M carbonate buffer at pH 10 (150 μl) and the soln was added to the crude acryloylated oligosaccharide **5** or **9** (4.3 μmol) in a 1 ml Eppendorf tube. The vial that contained the BSA solution was washed with more buffer (2 × 50 μl) and the washings were added to each reaction mixture (final vols: 200 μl) which were left 5–7 days at 37 °C. The glycoconjugates **13** and **14** were dialysed against distilled water (6 × 6 ml) using an Amicon ultrafiltration cell equipped with a Diaflo membrane. The residues were taken up in water and lyophilized to give **13** and **14** as white powders (11 mg in both cases). The carbohydrate contents of glycoconjugates **13** and **14** were 11 and 5 hapten molecules per BSA molecule, respectively (39% incorporation of the pentasaccharide and 18% incorporation of the hexasaccharide), as measured by the method of Dubois et al.[34] The unreacted acryloylated pentasaccharide and hexasaccharide were recovered by lyophilization of the ultrafiltrates followed by de-ionization of the residues with Rexyn 101 (H^+) resin and repeated lyophilizations from water to remove residual HCl. NMR spectra and TLC of the recovered penta- and hexasaccharides showed that they were pure enough to be used in further coupling reactions.

Preparation of the ovalbumin-glycoconjugates 15 and 16

Two samples of ovalbumin (10 and 5 mg) were each dissolved in a 0.1 M carbonate buffer at pH 10 (150 and 50 μl, respectively). The solns were added to the crude acryloylated oligosaccharides **5** (4.5 μmol) or **9** (2.1 μmol) in a 1 or 0.5 ml Eppendorf tube, respectively. The vials that contained the ovalbumin solutions were washed with more buffer aliquots and the washings were added to the reaction mixtures (final vols: 200 and 100 μl, respectively) which were left for 7 days at 37 °C. The glycoconjugates **15** and **16** were purified and isolated as white powders (10 and 5 mg, respectively) using the same conditions as described for the preparation of the glycoconjugates **13** and **14**. A sample of pure ovalbumin, tested by the method of Dubois et al.[34] showed a non-negligible amount of sugar. Therefore, the results obtained for the glycoconjugates **15** and **16** were corrected to give the amount of oligosaccharide incorporated in each reaction. The glycoconjugates **15** and **16** were shown to contain 1 pentasaccharide and 7 hexasaccharide haptens per ovalbumin molecule, respectively (5% incorporation of the pentasaccharide and 37 % incorporation of the hexasaccharide).

Preparation of the ovalbumin-glycoconjugate 17

The crude pentasaccharide **6** (4.6 μmol) was dissolved in a 0.1 M carbonate buffer pH 10 (100 μl) and added to an aliquot of ovalbumin (10.3 mg) in a 1 ml Eppendorf tube. The vial that contained **6** was washed with more buffer (2 × 50 μl) and the washings were added to the reaction mixture (final vol.: 200 μl) which was left 7 days at 37 °C. The reaction was worked-up as described for the preparation of glycoconjugate **13** and the glycoconjugate **17** was obtained as a white powder (6.9 mg after losses due to a broken vial). The pentasaccharide content of glycoconjugate **17** was 5 hapten molecules per ovalbumin molecule (26% incorporation of the pentasaccharide), as measured by the method of Dubois et al.[34] and after correction, as described for **15**.

Preparation of the ovalbumin-glycoconjugate 18

The crude pentasaccharide **6** (2.35 μmol) was coupled in a 0.1 M carbonate buffer pH 10 to a sample of ovalbumin (5.1 mg) using the same concentration conditions described for the preparation of glycoconjugate **17**. The reaction was left 7 days at room temperature and worked-up as described for glycoconjugate **13**. The glycoconjugate **18** was obtained as a white powder (8 mg) and its pentasaccharide content, 9 hapten molecules per ovalbumin molecule (45% incorporation

of the pentasaccharide), was measured in the same conditions than described for **15**.

Preparation of the BSA-glycoconjugate 19

The crude hexasaccharide **10** (2.13 μmol) was dissolved in a 0.1 M carbonate buffer pH 10 (50 μl) and added to a sample of BSA (5.4 mg) in a 1 ml Eppendorf tube. The vial that contained **10** was washed with more buffer (2 × 25 μl) and the washings were added to the reaction mixture (final vol.: 100 μl) which was left 7 days at room temperature. The reaction was worked-up as described for the preparation of glycoconjugate **14** and the glycoconjugate **19** was obtained as a white powder (8 mg). The glycoconjugate **19** was shown to contain 16 hexasaccharide haptens per BSA molecule, respectively (59% incorporation) as measured by the method of Dubois et al.

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